Supplementary file

Materials and methods

Patients and control subjects

A written-informed consent was obtained from patients (n = 5) and non-atopic subjects (n = 5) recruited at the Hospital Regional Universitario of Málaga (Spain, Table S1). Specific-IgE to Ole e 1 (t224) and Der p 1 (d202) were determined by ImmunoCAP-FEIA (Thermo Fisher Scientific, Uppsala, Sweden) in serum samples, according to manufacturer instructions. All patients were negative to Der p 1. Blood was collected by percutaneous venepuncture from subjects.

Proteins

Ole e 1 was isolated and purified from olive pollen (IBERPOLEN SL, Jaén, Spain) as previously described [1]. Endotoxin-free (≤ 0.03 EU/µg) natural Der p 1 (LTN-DP1-1, Lot. 38190) was supplied from Indoor Biotech (Cardiff, UK). In these experiments, Der p 1 was activated by incubation with 0.1 mM reduced glutathione (GSH, Sigma) in PBS at 37ºC for 15 min. Alternatively, cysteine-protease activity was checked by measuring the hydrolysis of the fluorogenic substrate N-tert-butoxycarbonyl-Gln-Ala-Arg-7-amido-4-methylcoumarin (Boc-QAR-AMC, Cat. No. BML-P237-0005, Enzo Life Sciences, NY, USA) in PBS containing 0.1 mM GSH on a microplate reader FLUORstar OPTIMA (BMG LabTech, Ortenberg, Germany) at 355 nm excitation and 460 nm emission wavelength, for 90 min (data not shown) [2].

Cell line and air-liquid interface cultures

Human bronchial epithelial Calu-3 cells (3 x 10^6 cells/cm^2, passages 23-30, American Type Culture Collection, Barcelona, Spain) were cultured onto 6.5 mm-transwell inserts

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(pore size 0.4 µm, Corning 3470), at air-liquid interface (ALI) in DMEM nutrient mixture F12 (Thermo Fisher Scientific, MA, USA) supplemented with 2 mM L-glutamine (Sigma), 100 U/mL penicillin, 100 µg/mL streptomycin (Lonza, Basel, Switzerland) and 5% foetal bovine serum (FBS, Hyclone GE Healthcare, Little Chalfont, UK), at 37ºC and 5% CO_2. ALI-cultured Calu-3 cells were exposed apically to 0.1 mL of the Ole e 1 (25 µg/mL) and/or Der p 1 (10 µg/mL) in PBS containing 0.1 mM GSH for different time points on days 2 and 7, adding 0.5 mL of complete DMEM-phenol free-medium (Thermo Fisher Scientific) with 5% FBS to the basolateral side. Cells treated with PBS were used as control.

The establishment of the Calu-3 cell barrier was checked by measuring the transepithelial electrical resistance (TEER). ALI-cultured Calu-3 showed a gradual increased in TEER values, reaching a plateau (600 Ω.cm*2*) at day 7, which is maintained until day 14(Figure S1). On day 2 at ALI, the epithelial barrier was still forming as indicated by the low TEER values (under 100 Ω.cm2). In contrast, TEER values above 600 Ω.cm^2 were indicators of the establishment of epithelial barrier by day 7 at ALI. Thus, to study how the functional state of the epithelial barrier influences the response to Ole e 1, days 2 and 7 were selected according to the kinetic of the barrier establishment.

Transepithelial electrical resistance monitoring

TEER was monitored using an EVOM voltmeter device (World Precision Instruments, Florida, U.S.A.) with an STX2 chopstick electrode, according to the manufacturer's guidelines. For each condition, six individual transwell inserts were used, and triplicate measurements were performed for each well. TEER value (Ω.cm^2) was calculated by subtracting the average resistance of cell-free transwell inserts and by multiplying by the effective growth area (0.33 cm^2).

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Epithelial permeability determination

Epithelial permeability to Ole e 1 was analysed by immunoblotting using a rabbit polyclonal anti-Ole e 1 antiserum (1/5000, generated by Dr. F. Vivanco's laboratory at the Fundación Jiménez Díaz, Madrid, Spain), as previously described [3].

Immunofluorescence labelling and confocal laser scanning microscopy of tight junctions

After washing with PBS, ALI-cultured Calu-3 cells were fixed with 4% (w/v) paraformaldehyde (Sigma) in PBS for 15 min at room temperature, permeabilized with 0.05% (v/v) Triton-X100 (Sigma), and blocked with 3% (w/v) bovine serum albumin (BSA, Sigma) in PBS containing 0.05% Tween-20 (Sigma) for 1 h. Rabbit polyclonal anti-ZO-1 (1/75, Invitrogen) was used as primary antibody for TJs subcellular localization, and a goat anti-rabbit IgG labelled with Alexa 647 (1/400, Invitrogen, CA, USA) as secondary antibody, both incubations were performed in PBS containing 0.05% (v/v) Tween-20 and 0.05% (w/v) BSA for 1 h at room temperature. After staining, membranes were removed from transwell inserts using a sterile scalpel and mounted onto glass slides (Deltalab, Barcelona, Spain) with ProLong® Gold antifade mountant containing DAPI (4',6-Diamidino-2-Phenylindole Dihydrochloride, Thermo Fischer Scientific). Images were obtained with an OLYMPUS FV1200 confocal laser microscope at the Centro de Citometría y Microscopía de Fluorescencia (CAI-UCM). Experiments were performed at least in duplicate and representative images are shown.

Protease activity of Der p 1 on Ole e 1

Ole e 1 was incubated *in vitro* with pre-activated (15 min, 0.1 mM GSH) Der p 1 (1:1, protein/enzyme ratio, w/w) in PBS containing 0.1 mM GSH at 37ºC for different time-

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points (0, 0.5, 8 and 24 h). Then, the reaction was stopped by addition of 100 µM of the specific inhibitor E-64 (Sigma-Aldrich), and samples were analysed by matrix-assisted laser desorption-ionisation mass spectrometry (MALDI-TOF-MS) on an Autoflex III MALDI-TOF-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with a smart beam laser, in the positive ion detection and delayed extraction linear mode. Typically, 1000 laser shots were summed into a single mass spectrum. External calibration was performed, using Protein Calibration Standard I (Bruker), covering the range from 5000 to 20000 Da. A matrix solution of saturated α-cyano-4-hydroxycinnamic acid in acetonitrile/0.1% trifluoro-acetic acid was used at a ratio of 1:2 of sample to a matrix, and 1 µL of this mixture was spotted on the 800 µm Anchor Chip target (Bruker-Daltonics, MA, USA). The molecular mass analysis by MALDI-TOF-MS was carried out in Proteomics and Genomics Facility (CIB-CSIC), a member of ProteoRed-ISCIII network.

In addition, 0.5 h-digested Ole e 1 was submitted to N-terminal sequencing analysis. Samples were incubated at 90ºC for 10 min before loading on 15% SDS-PAGE, electroblotted onto polyvinylidene difluoride membrane (Immobilon-P, Millipore, MA, USA) and stained with Coomassie Brilliant Blue. Proteins bands were excised from the membrane and N-terminal Edman degradation was carried out using a Procise 494 HT Sequencing System (Applied Biosystems, Foster City, CA, USA) at the same facility.

ELISA inhibition assay

ELISA inhibition was performed as previously described [4]. Briefly, serum of each allergic patient (n = 3, 1/10 diluted) was preabsorbed with inhibitors in PBS-Tween 20 0.5% (v/v) containing 3% (w/v) milk for 2 h at room temperature, and then added to Ole e 1 (1 µg/mL)-coated plate wells (Costar) and incubated for 2 h at 37ºC. Ole e 1-cleavage products (>3 kDa and <3k Da) were separated by Nanosep®-3K (Pall Corporation) and used as inhibitors (1 µg/mL). Equivalent amounts of non-treated and

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HCl-hydrolysed Ole e 1 were used as positive and negative controls, respectively. Bound IgE antibodies were detected using o-phenylenediamine in 0.05 M phosphate citrate buffer containing H_2O_2, and optical density (OD) was read at 492 nm on an iMark Microplate Absorbance Reader (Bio-Rad). The percentage of inhibition was calculated according to the formula: % Inhibition = [1 – (OD492 nm with inhibitor/OD492 without inhibitor)] x 100. Alternatively, a rabbit polyclonal anti-Ole e 1 (1/5000) was used in the ELISA inhibition experiments for IgG-binding determination.

Basophil activation assay

Whole blood samples from Ole e 1-sensitized patients and non-atopic individuals (100 µl) were incubated with 20 µl of stimulation buffer [1M HEPES buffer containing 0.78% NaCl (w/v), 0.037% KCl (w/v), 0.078% $CaCl_2$ (w/v), 0.033% $MgCl_2$ (w/v) and 0.1% human serum albumin (w/v)] 0.2 µg of IL-3 (R&D Systems) and stained with 1 µg of anti-CCR3-APC-conjugated antibody (BioLegend INC, San Diego, USA) for 10 min at 37ºC. Then, cells were incubated with 100 µl of sample serial dilutions (1/50-1/10000 and 1/5-1/10000 for >3 and ≤3 kDa fractions, respectively) for 30 min at 37ºC. PBS and anti-human IgE (0.5 mg/ml, BD Biosciences, NJ, USA) were used as negative and positive controls, respectively. The degranulation process was stopped by incubation 5 min on ice, and cells were analyzed by three-color flow cytometry (FACSCalibur flow cytometer, BD Biosciences) using anti-CD203c-PE (BioLegend) and anti-CD63-FITC (BioLegend). The acquisition was performed on at least 500 basophils per sample, and results were expressed as the percentage of basophils expressing CD63 (CD63+CD203c+CCR3+).

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References

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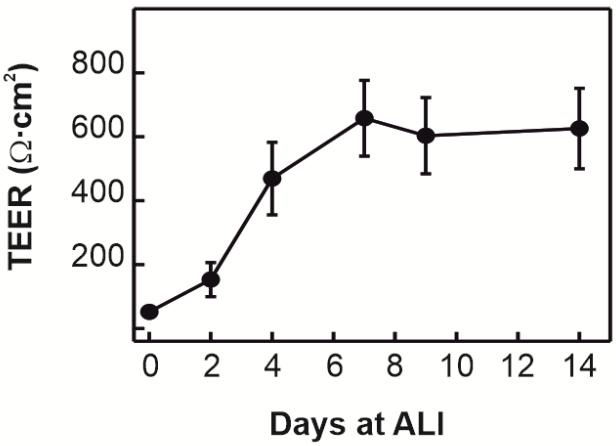
4. Huecas S, Villalba M, Gonzalez E, Martinez-Ruiz A, Rodriguez R. Production and detailed characterization of biologically active olive pollen allergen Ole e 1 secreted by the yeast *Pichia pastoris*. Eur J Biochem 1999;261:539-6.

Supplementary Figures and Tables

Supplementary Figure 1. Time course of TEER values of ALI-cultured Calu-3 cells.



TEER was measured at the indicated time points, and shown as the mean value ± SD of triplicate determinations.

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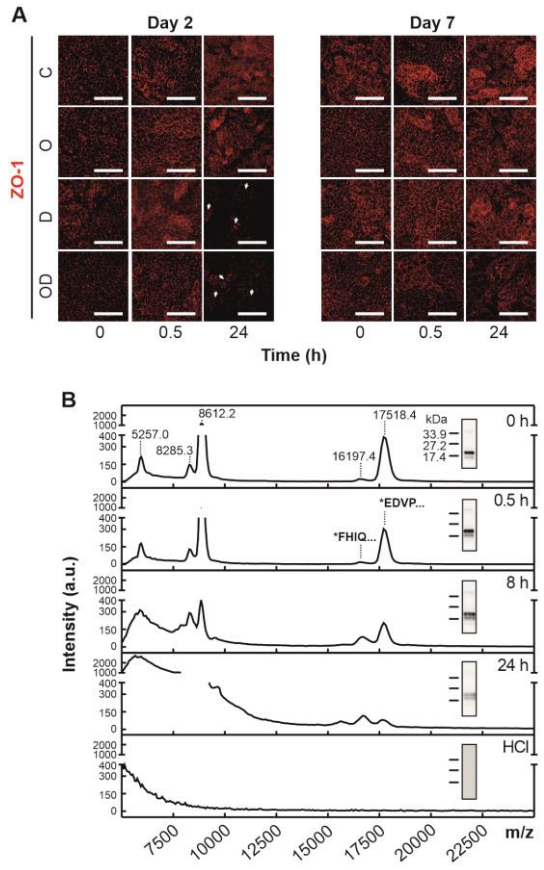
Supplementary Figure S2. Effect of Der p 1 co-exposure on the barrier integrity of ALI-cultured Calu-3 cells on days 2 and 7, and on Ole e 1 allergen.



A) Confocal laser scanning microscopy analysis of ZO-1 (red) expression over time of exposure. Representative Z-stack projection of 12-16 individual sections are shown. White arrows indicate TJ disruptions. Scale bar = 25 µm. C, non-treated cells; O, cells exposed to Ole e 1; D, cells exposed to Der p 1 protease; OD, cells co-exposed to the combination of Ole e 1 and Der p 1.

B) MALDI-TOF-MS analysis of Ole e 1-cleavage products by Der p 1 at different time points. The mass/charge ratio (m/z) is shown: monoprotonated (16197.4 and 17518.4 m/z) and bi-protonated (8285.3 and 8612.2 m/z) species of non-glycosylated and glycosylated of Ole e 1 forms, respectively. The N-terminal sequences of full- and short-length forms of Ole e 1 protein (*) obtained by Edman degradation after protease-treatment for 0.5 h are shown. Insert, immunoblotting of Ole e 1 using a specific polyclonal antiserum over 24 h of protease-treatment.

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Molecular mass protein standards in kDa are indicated. Ole e 1 hydrolysed with 6N HCl was used as a negative control: HCl.

Supplementary Table 1. Clinical data of the individuals used in the basophil activation assays.

Nº*	Diagnosis	Symptoms	Smokers	Gender†	Age‡	sIgE§
P1	Persistent seasonal severe RC	Nasal obstruction; sneezing; runny nose; naso-ocular itching; ocular redness; lachrymation	No	M	28	1.93
P2	Intermitent seasonal moderate RC	Sneezing; runny nose; naso-ocular itching; lachrymation	Ex (5 years)	F	36	18.4
P3	Persistent permanent moderate RC	Intermittent nasal obstruction; sneezing; runny nose; naso-ocular and oral itching; lachrymation	Ex (5 years)	F	58	1.55
P4	Persistent seasonal moderate RC and asthma	Sneezing; runny nose; naso-ocular itching; tearing; dyspnea	No	M	44	17.2
P5	Persistent seasonal moderate RC and asthma	Nasal obstruction; sneezing; runny nose; naso-ocular itching; dyspnea; cough	Yes	F	48	1.07
C1	ND	ND	No	M	26	<0.35
C2	ND	ND	No	M	69	<0.35
C3	ND	ND	No	F	49	<0.35
C4	ND	ND	No	F	38	<0.35
C5	ND	ND	No	M	29	<0.35

*No., Donor operative number: P, allergic patient; C, non-atopic individual.
†Gender: M, male; F, female.
‡Age in years.
§sIgE, specific IgE levels (kU/L) for Ole e 1. For all donors sIgE for Der p 1 were < 0.35 kU/L.
ND, non-described symptoms; RC, rhinoconjunctivitis